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Abstract

There is a growing interest in donkey's milk as food for sensitive consumers, such as infants with cow's milk protein allergy and elderly people. The aim of this study was to carry out a survey on the dairy donkeys farming in Piedmont, Italy. The research was conducted in order to analyze the farm characteristics as well as the chemical and microbiological quality of milk. All the farms were small-sized, family-run, and, in most cases, animals were farmed semi-extensively. The donkey milk from Piedmont farms was characterized by a protein content around 1.5 g/100 mL and a fat content lower than 0.1 g/100 mL. Lysozyme activity was considerably higher than that reported in raw cow milk. The milk microbiological profile greatly differed among the farms. Milk sampled in the farm that performed hand milking showed total viable counts significantly lower than milk collected in the farms equipped with automatic milking. Samples were tested for several pathogens and negative results were observed, except for the detection of *B. cereus* in one sample. The survey provided useful data for the laying down of recent regional regulation for the production and commercialization of donkey's milk. The results of the survey indicate that further research is needed in order to define the best management and nutritional strategies for the improvement of the quali-quantitative production of dairy donkeys.

Key words: donkey farming, donkey milk, microbiological quality, protein profile, lysozyme

Abbreviations:

DM: donkey milk; LAB: lactic acid bacteria; PVDF: polyvinylidene fluoride; TVC: total viable count.

1. Introduction

There is a growing interest in donkey farming for milk production, since donkey's milk (DM) is considered a valid alternative foodstuff in terms of clinical tolerability, palatability and nutritional adequacy for children affected by cow's milk protein allergy (Iacono et al., 1992; Mansueto et al., 2013; Monti et al., 2007; Monti et al., 2012).

Since 2005, the number of published papers on DM has increased to almost 30 per year, the great majority by Italian researchers. Although data covering the potential of DM use as a substitute of cow's milk for allergic individuals are available, information regarding donkey farm management and some aspects of milk production are limited, such as the study of its main microbial communities. The majority of surveys on DM quality were carried out in the South of Italy, mainly on Martina Franca (D'Alessandro, De Petro, Claps, Pizzillo, & Martemucci, 2009; D'Alessandro & Martemucci, 2007; D'Alessandro, Martemucci, Jirillo, & Leo, 2011; Martemucci & D'Alessandro, 2012) and Ragusana breeds (Alabiso, Giosuè, Alicata, Mazza, & Iannolino, 2009; Giosuè, Alabiso, Russo, Alicata, & Torrisi, 2008). One very recent report was published on Amiata donkey milk chemical composition (Martini, Altomonte, & Salari, 2014). Some data are also available for Chinese donkeys (Guo et al., 2007) and for Balkan Littoral-Dinaric breed (Ivanković et al., 2009; Šarić et al., 2012). The literature indicates that fat content in DM ranges from 0.3 to 1.8 g/100 mL, while the protein content is reported to be less variable, with values from 1.4 to 1.8 g/100 mL (Medhammar et al., 2012). DM is also known to contain high concentration of lysozyme, if compared to other mammalian milks. Donkey's lysozyme, similarly to cow's, displays antibacterial activity against a vast number of bacteria (Conte, Foti, Malvisi, Giacopello, & Piccinini, 2012; Zhang, Zhao, Jiang, Dong, & Ren, 2008; Šarić et al., 2012), affecting directly the composition of the milk microbiome (Sarno, Santoro, Di Palo, & Costanzo, 2012). Very recently, it has been shown that the addition of jenny milk during cheese-making reduced the number of coliforms in the resulting cheese (Cosentino, Freschi, Paolino, & Valentini, 2013). Further compositional aspects of

DM, that are not considered in the present study, were extensively reviewed in two recent reports by Salimei & Fantuz (2012) and Medhammar and coworkers (2012).

Low bacterial counts are generally reported in DM, in comparison to milk and dairy products from other animals (Pilla, Daprà, Zecconi, & Piccinini, 2010; Salimei et al., 2004; Sarno et al., 2012). Previous reports have shown that the DM microbiota is mainly composed by lactic acid bacteria (Zhang et al., 2008; Šarić et al., 2012). Nevertheless, undesirable species bacteria, such as *Streptococcus* spp., *Staphylococcus* spp. and coliforms, have been also detected in DM (Pilla et al., 2010; Sarno et al., 2012).

The aim of this research is to provide the first overview of the chemical and microbiological characteristics of milk produced in dairy donkey farms in the North West of Italy. This outline provided a significant contribution to the recent publication of a regional regulation for the production and commercialization of DM (Regione Piemonte Direzione Sanità Settore Prevenzione e Veterinaria, 2013).

2. Materials and Methods

2.1. Farm description and milk sampling

Five donkey dairy farms located in the North Western Italy, in Piedmont, were surveyed during this study, and a questionnaire was filled in for each farm. Data and samples were collected during Autumn 2012 and Spring 2013. The on-farm survey included a face-to-face interview with the farm manager. We developed a questionnaire, which consisted of semi-closed questions, including topics related to farm management and husbandry practices.

The DM samples were obtained from a different number of jennies and by different milking practices, depending on farm management. In each surveyed farm, one liter of DM was sampled for

chemical and biochemical analyses, while six replicate DM aliquots (300 mL each) were collected in sterile bottles for microbiological screening.

Immediately after collection, DM samples were transported, refrigerated and either used fresh (as for microbiological analyses), stored within 6 hours from the collection at -20 °C, or lyophilized.

2.2. Milk chemical and biochemical analyses

The DM samples were analyzed for gross energy, dry matter, crude protein and ether extract contents. In addition, the lysozyme activity of each sample was determined using a fluorescence-based assay (EnzCheck® Lysozyme Assay Kit, Life Technologies Italia, Monza, Italy).

Dry matter content was measured on DM using a gravimetric method (Baldini, Fabietti, Giammarioli, Onori, Orefice, & Stecchini, 1996). Gross energy content was determined in excess oxygen by adiabatic bomb calorimeter (Mod. 700, IKA GmbH & Co., Staufen, Germany), using benzoic acid as a reference (26.454 MJ/kg). The nitrogen content in milk samples was estimated by Kjeldahl-based block digestion method (AOAC Official Method 991.20, 2000), using a 2020 Tecator Digestor (VWR International Pbi, Milano, Italy), a Kjeltec-System 1002 Distilling Unit (Foss Italia, Padova, Italy) and a 655 Dosimat automatic titrimeter (Metrohm Italiana, Origgio, Italy). The total fat content of DM samples was measured gravimetrically on ether extract using a Soxhtraction device (VWR International Pbi), following manufacturer's instructions. All the analyses were performed on lyophilized DM samples in triplicate.

Lysozyme activity assay was conducted by EnzCheck® Lysozyme Assay Kit following manufacturer's instruction in triplicate on 500-fold diluted DM samples, using a fluorescence microplate reader (Victor 3D, Perkin Elmer, Waltham, USA), equipped with fluorescein filters (485 nm emission and 535 nm excitation).

2.3. Milk protein profile

DM samples were accurately mixed and vortexed at room temperature in order to ensure homogeneity, then 100 µL from each sample were taken up to 1 mL with milliQ water and vortexed thoroughly again. One aliquot (100 µL) of each diluted sample was mixed with the same volume of NuPAGE® LDS Sample Buffer (2X diluted) (Life Technologies Italia), containing 50mM dithiothreitol (Sigma Aldrich, Milano, Italy) as reducing agent, and warmed to 70°C for 10 minutes. Each sample solution (10 µL) was then loaded onto a 10-well, NuPAGE® Bis-Tris mini gels (12% polyacrylamide, 1mm width). One lane on each gel was loaded with 5 µL of Mark12® Unstained Standard (Life Technologies Italia).

The proteins were separated on a Novex Mini-cell (Life Technologies Italia) filled with cold 1X NuPAGE® MES SDS Running Buffer (Life Technologies Italia), at 200V. The gels were then stained with colloidal Coomassie Blue staining (Candiano et al., 2004) and digitized with a ImageScanner device (Amersham Pharmacia, now GE Healthcare Life Sciences, Uppsala, Sweden) at 300 dpi.

Two bands were excised with a sterile scalpel and passively eluted from the gel pieces onto PVDF membranes, as previously described (Reuter et al., 2005) to be subjected to N-terminal sequencing. The membranes were then microsequenced on a Procise 492 protein sequencer (Applied Biosystems, now Life Technologies Italia). All the chemicals used in the procedure were from Life Technologies Italia. The N-terminal amino acid sequences were searched with the MS-Homology software package on NCBI non-redundant database (NCBInr2013.6.17).

2.4. Milk microbiological analyses

For this part of the study, a third sampling was carried out during the 2014 Spring season.

Two aliquots of each DM sample were collected and stored in sterile conditions at 4°C. One of them was analyzed the day after milking, while the second one was examined 5 days after the sampling.

The total viable count (TVC) was performed onplate count agar, using the inclusion method. One mL of undiluted samples and of each 10^{-1}, 10^{-2} and 10^{-3}dilutions in a mixture of physiological saline solution and peptone (85:15 v:v) (OXOID LTD, Basingstoke, Hampshire, England) were included in the plate count agar (OXOID LTD) and plates were incubated for 72 hours at 30ºC before counting. The results were then expressed as cfu/mL (UNI EN ISO 4833: 2004). Lactic acid bacteria (LAB) colonies, grown on MRS agar (OXOID LTD) at 30ºC for 48 hours in anaerobiosis were counted (ISO/FDIS 15214:1998). According to ISO 6579:2002, for the detection of *Salmonella* spp., BPW enrichment media (OXOID LTD) was used for an incubation of 24 hours at 37ºC, a second incubation was performed with Rappaport media (OXOID LTD) (41ºC for 24 hrs) and with MKTTn (OXOID LTD) at 37ºC for 24 hrs. Both incubation products were finally grown on XLD agar medium (OXOID LTD) for 24 hrs at 37ºC.

For *E. coli,* EN/ISO 16649/2:2001 regulation parameters were followed, growing in TBX agar (OXOID LTD) at 44ºC for 24 hrs. Regarding *E.coli* O157, the enrichment media TSBm (OXOID LTD) was utilized, incubated at 41ºC for 24 hours. Dynabeads anti-*E. coli* O157 (Invitrogen Dynal AS, Oslo, Norway) were used to selectively capture these bacteria. *E. coli* O157 was grown on TBX agar (OXOID LTD) at 41°C for 24 hrs and on CT-SMAC (OXOID LTD) at 37°C for 24 hrs.

Listeria monocytogenes presence was evaluated by enriching with Demi-Fraser (Sifin, Berlin, Germany) and Fraser media (Sifin) incubated at 37ºC for 24 hours (EN/ISO 11290/1:1996), a subsequent growth on OCLA and PALCAM agar mediums (OXOID LTD) was done for both enriching media for 48 hrs at 37 ºC.

For *B. cereus*, the Bacillus agar base medium (OXOID LTD) was used, incubating at 37ºC for 24 hrs (EN/ISO 7932:2005).

The detection of *Campylobacter* spp, (Bacteriological Analytical Manual - FDA (2001)) consisted in an initial enrichment with Bolton Broth media (OXOID LTD), an incubation of 41ºC for 24 hours in anaerobiosis and a final growth on CCD agar (OXOID LTD) in the same conditions but for 48 hrs.

All the analysis were done in technical triplicate.

2.5. Statistical analysis

Significant differences in total viable count (log10 cfu/mL) among the farms were assessed by ANOVA at $p \leq 0.05$, and classes of uniformity were grouped according to Tukey's post-hoc test. Significant differences in lactic acid bacteria count (log10 cfu/mL) among the farms were assessed by Kruskal-Wallis non parametric test at $\alpha \leq 0.05$, and classes of uniformity were grouped according to Dunnet-C post-hoc test, not assuming equal variances. The statistical significance of increase in TVC and LAB counts from day 0 to day 5 were evaluated by Student T test (not shown).

2.6 PCR for confirmation of Bacillus cereus

DNA was extracted by boiling procedure from broth and amplified as previously reported (Park, Kim, Kim, Kim & Kim, 2007). The PCR product was visualized in a 2% agarose gel (Invitrogen, Carlsbad, USA) in the Gel Doc XR+ system (BIO-RAD Italia, Segrate, Italy).

3. Results and discussion

3.1. Farm characteristics

Data regarding farm management and husbandry practices are reported in Table 1. All the farms were small-sized, family-run, and recently began the donkey farming activity. In most cases, animals were farmed semi-extensively, partially grazing and partially fed *ad libitum* with on-farm produced hay. Farms A, C and E were located in agricultural areas. Farm B was located in a mountain rural area. Farm D, which was located in a hilly area, did not produce or sell milk during the surveyed timeframe. In all the farms surveyed, donkeys spent around 12 h/d on pasture. All farms, except one, used machine milking and milked the jennies once a day. The mean milk yield

was around 1 L/day/animal. All the farms sold the milk directly to families of cow's milk protein allergic children. Some of the producers also allocated part of the milk for cosmetic production.

3.2. Donkey milk chemical characterization

The average quality of DM produced in Piedmont farms was assessed by evaluating its chemical characteristics, in Autumn and Spring. Table 2 reports the mean values recorded for each assayed parameter in the two different samplings, as well as their overall means. Milk was characterized by a mean protein content around 1.5 g/100mL and a mean fat content lower than 0.1 g/100mL. Farm C milk was the only sample displaying a slightly higher fat concentration, ranging from 0.2 to 0.4 g/100mL, depending on the season. Fat is the most variable component of DM, ranging from 0.1 g/100mL (Salimei et al., 2004) to 1.8 g/100mL (Guo et al., 2007) in different reports, being affected from both lactation and foaling season (Cosentino et al., 2013; Giosuè et al., 2008; Ivanković et al., 2009; Salimei et al., 2004). The Piedmontese DM showed very low fat concentration, even below the lower limit reported in literature. The low DM fat concentration and its consequent low energy content are the main limits to its use in nutrition of children allergic to cow's milk protein, in their first year of life, since recommended dietary allowances may not be reached, unless adequate supplementation is provided (D'Auria, Mandelli, Ballista, Di Dio, & Giovannini, 2011). Future perspectives on DM research thus include studies aimed at setting up new nutritional strategies for the lactating donkey, in order to increase the DM energetic value. On the other hand, this feature makes DM an hypo-caloric and highly digestible food for consumers with specific dietary requirements, such as athletes and elderly people.

The observed values for milk protein content are in accordance to the results reported for Italian DM. Milk was reported to range between 1.2 g/100mL (Cosentino et al., 2013) and 1.9 g/100mL (Giosuè et al., 2008), and it has been shown to be affected by the lactation stage, the lactation and foaling season (D'Alessandro et al., 2009; Giosuè et al., 2008; Ivanković et al., 2009; Martemucci & D'Alessandro, 2012; Salimei et al., 2004; Tidona et al., 2014).

The DM produced in Piedmont farms was characterized by a mean lysozyme activity of 162 U/μL, very high in comparison to other dairy species, as also reported by Conte et al. (2012). Such an elevated lysozyme activity may explain the low incidence of mastitis in jennies, that usually follows physical injuries to the glands or drying off (Conte, Piccinini, Daprà, & Gagliano, 2006).

3.3. Donkey milk protein profile characterization

The mono-dimensional gradient electrophoresis pattern of the proteins contained in milk samples from the different farms is reported in Figure 1. The NuPAGE® gels coupled with MES SDS Running Buffer were chosen in order to enhance the separation of medium-low molecular weight proteins, and to allow a reliable and easy comparison of the protein patterns from the DM samples collected in the different farms. Small differences in the profile of the different DM samples could be seen. Milk collected from Farm B displayed a slightly different protein pattern, showing two protein bands, characterized by an apparent different abundance compared to the other milk samples, especially in the Spring sampling. The two protein bands from Farm B milk sample were subjected to N-terminal sequencing in order to be identified. Results are detailed in Table 3. The two bands represented a mixture of beta lactoglobulin 1 and alpha s2 casein B (band 1) and beta lactoglobulin 1 (band 2). Differential protein glycosylation of beta lactoglobulin and phosphorylation of alpha s2 casein, already observed on the same proteins in previous DM proteomic characterization (Bertino et al., 2010), may play a role in the observed changes in protein bands. It is interesting to note that Farm B was the only farm breeding exclusively Martina Franca donkeys. Polymorphism of protein isoelectrofocusing bands was already observed in Ragusana breed by Criscione et al. (2009), which reported the absence of other DM proteins (alpha s1 casein and beta lactoglobulin II) in some individuals. They also reported the absence of alpha s2 casein in all DM samples. In a very recent study, Tidona et al. (2014) investigated the relevance of DM protein profile individual variability in determining the size of casein micelles and *in vitro* milk protein digestibility. They reported that the size of casein micelles was not modified by the absence

of alpha s1 casein, while it was partially correlated with total protein content. They also reported that the absence of beta lactoglobulin II increased the rate of gastric digestion of the lactoglobulin fraction in DM. As alpha casein and beta lactoglobulin isoforms are considered as major allergens in cow milk, the presence/absence of these protein bands may have an impact on the residual allergenic potential of DM, as already reported by Tidona et al. (2014). A deeper insight into DM protein profiles from different breeds may shed light on this feature.

3.4. DM microbiological quality

The occurrence of known milk pathogens in DM was investigated by searching for *Salmonella* spp., *E. coli*, *E. coli* O157, *Listeria monocytogenes*, *Bacillus cereus* and *Camplylobacter* spp. The results were negative for all the farms at day 0 as well as at day 5, with the exception of farm A in the 2014 Spring sampling, being positive only for B. cereus (1.3*102 cfu/mL). The presence of B. cereus in milk has been previously reported, with seasonal fluctuations in raw and processed cow milk (Bartoszewicz, Hansen, & Swiecicka, 2008). Preliminary studies on DM have shown the presence of *B. cereus*, with counts similar to the ones found in this study (Scatassa et al., 2011).

The total viable and lactic acid bacteria counts obtained for each of the farms on the three samplings are shown in Table 4. The Farm A presented the highest TVC in this study (Table 4). Different microbiological tests and sequencing analyses were performed for some isolates of this farm (data not shown), showing *Pseudomonas* spp. as the group having the highest occurrence, thus suggesting a possible contamination issue.

On the other hand, farm D presented the lowest values for the TVC (Table 4). This farm was run extensively. The animals were free to pasture and were hand milked, not regularly, in contrast with the standard management of the other surveyed farms (Table 1). Moreover, Farm D displayed the lowest TVC value after 5 days, in contrast to farms using automatic milkers, also being the only farm to show a not significant increase of TVC following cold storage, after Student T test comparison of data at day 0 and day 5 (data not shown). Similar results were observed in a previous

study on goat milk in Spain (Delgado-Pertiñez et al., 2003): the authors found that hand milking determined a significant reduction of bacteria in the milk, in comparison to farms that use mechanic procedures. This observation might be due to the fact that hand milking results in a more suitable control of the hygienic management of the farm, as well as of milk collection and conservation. It is also important to mention that in all the studied farms, the milkers were adaptations of machines for goats or cows.

The overall mean for the three samplings of the TVC at day 0 was $2.4*10^5$cfu/mL. This value is one order of magnitude higher than previously reported (Chiavari et al., 2005; Coppola et al., 2002; Salimei et al., 2004; Zhang et al., 2008). Regarding the LAB count, the overall mean number was $1.6*10^4$ cfu/mL (Table 4). This LAB value is consistent with counts recently reported by Carminati et al. (2014) in DM. The LAB count increase in DM during cold storage was not significant, as assessed by Student T test comparison of data at day 0 and day 5 (data not shown), for any of the surveyed farms, thus indicating a possible effect of lysozyme antimicrobial activity against LAB. Gram-positive bacteria, such as LAB, possess a cell wall that can be hydrolyzed by lysozyme (Salazar & Asenjo, 2007), thus causing inhibition of LAB development and, as a consequence, decreasing their abundance in milk.

4. Conclusion

The profile of the donkey milk collected in Piedmont resulted to be quite similar among the surveyed farms, from a chemical point of view. The milk was characterized by a very low fat content, which may indicate the need for implementation of new nutritional protocols for the lactating donkeys, to increase its energetic value. The microbiological content of milk showed significant variations among surveyed farms, indicating a possible effect of the adopted milking practice. A better training of the farm operators is needed for an appropriate and efficient use of the

milking machinery. Besides, there is a need for the implementation of specific instruments to milk donkeys. An improvement in these aspects might help in reducing milk contamination, increasing the hygienic and sanitary properties of donkey milk.

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Figure captions

Figure 1. NuPAGE® (12% Bis –Tris; MES buffer) total protein profile of donkey milk, sampled from different farms (A-E) in the North Western Italy, in different sampling seasons (Panel A: Autumn; Panel B: Spring). Numbers: band 1 and band 2 (see Table 3 for identification). Colloidal Coomassie Brilliant Blue

458 stained. Std: Mark12® Unstained Standard markers (Life Technologies). Gels were run in reducing and
459 denaturing conditions.

Table 1. Farm characteristics as from face-to-face questionnaire to farm managers

	Farm A	**Farm B**	**Farm C**	**Farm D**	**Farm E**
Farm area (ha)	35	12	10	10	42
Altitude above sea level (m)	194	1.110	395	600	183
Total donkeys [a] ***(no.)***	65	63	45	150	48
Jennies [a] ***(no.)***	45	40	40	70	32
Milking jennies [a] ***(no.)***	7-10	7-10	8-10	30-33	6
Herd breed	Crossbreds	Martina Franca	Crossbreds	Crossbreds	Martina Franca, Ragusana, crossbreds
Total milk sold (L/year)	1200	2000	2000	0	1000
Milking practice	Automatic in milking room	Automatic in milking room	Automatic in cowshed	Hand milking	Automatic in milking room
Farming type	Semi-extensive	Semi-extensive	Semi-extensive	Extensive	Semi-extensive
Feed	Grazing - Hay	Hay - Bread – Protein supplementation	Grazing - Hay	Grazing - Hay	Grazing - Hay
Milk use	Food -cosmetics	Food	Food -cosmetics	-	Food -cosmetics

[a] counted during the visits

Table 2. DM chemical composition. Values represent mean values ± standard deviations (n=3), and the overall means of data collected in the surveyed farms.

	Season	Dry matter (g/100mL)	Crude proteins (g/100mL)	Total lipids (g/100mL)	Gross energy (kcal/100mL)	Lysozyme activity (U/µL)
Farm A	*Autumn*	8.77 ± 0.03	1.35 ± 0.04	0.02 ± 0.01	32.7 ± 0	166 ± 21
	Spring	8.69 ± 0.01	1.49 ± 0.02	0.01 ± 0.00	35.1 ± 0	140 ± 29
Farm B	*Autumn*	8.79 ± 0.02	1.41 ± 0.09	0.02 ± 0.01	35.5 ± 0	180 ± 23
	Spring	9.18 ± 0.03	1.53 ± 0.01	0.06 ± 0.00	37.9 ± 0	153 ± 28
Farm C	*Autumn*	9.80 ± 0.01	1.68 ± 0.01	0.40 ± 0.03	44.2 ± 0	181 ± 21
	Spring	8.87 ± 0.02	1.55 ± 0.01	0.22 ± 0.01	36.8 ± 0	194 ± 32
Farm D	*Autumn*	8.75 ± 0.02	1.75 ± 0.08	0.06 ± 0.02	35.7 ± 0	154 ± 34
	Spring	9.05 ± 0.00	1.39 ± 0.00	0.08 ± 0.01	38.0 ± 0	154 ± 13
Farm E	*Autumn*	8.35 ± 0.03	1.28 ± 0.02	0.02 ± 0.01	33.6 ± 0	155 ± 25
	Spring	8.72 ± 0.00	1.40 ± 0.06	0.02 ± 0.00	34.4 ± 0	140 ± 19
Overall	***mean***	8.89	1.48	0.09	36.3	162

Table 3. N-terminal sequencing and protein identification of bands 1 and 2 from Figure 1.

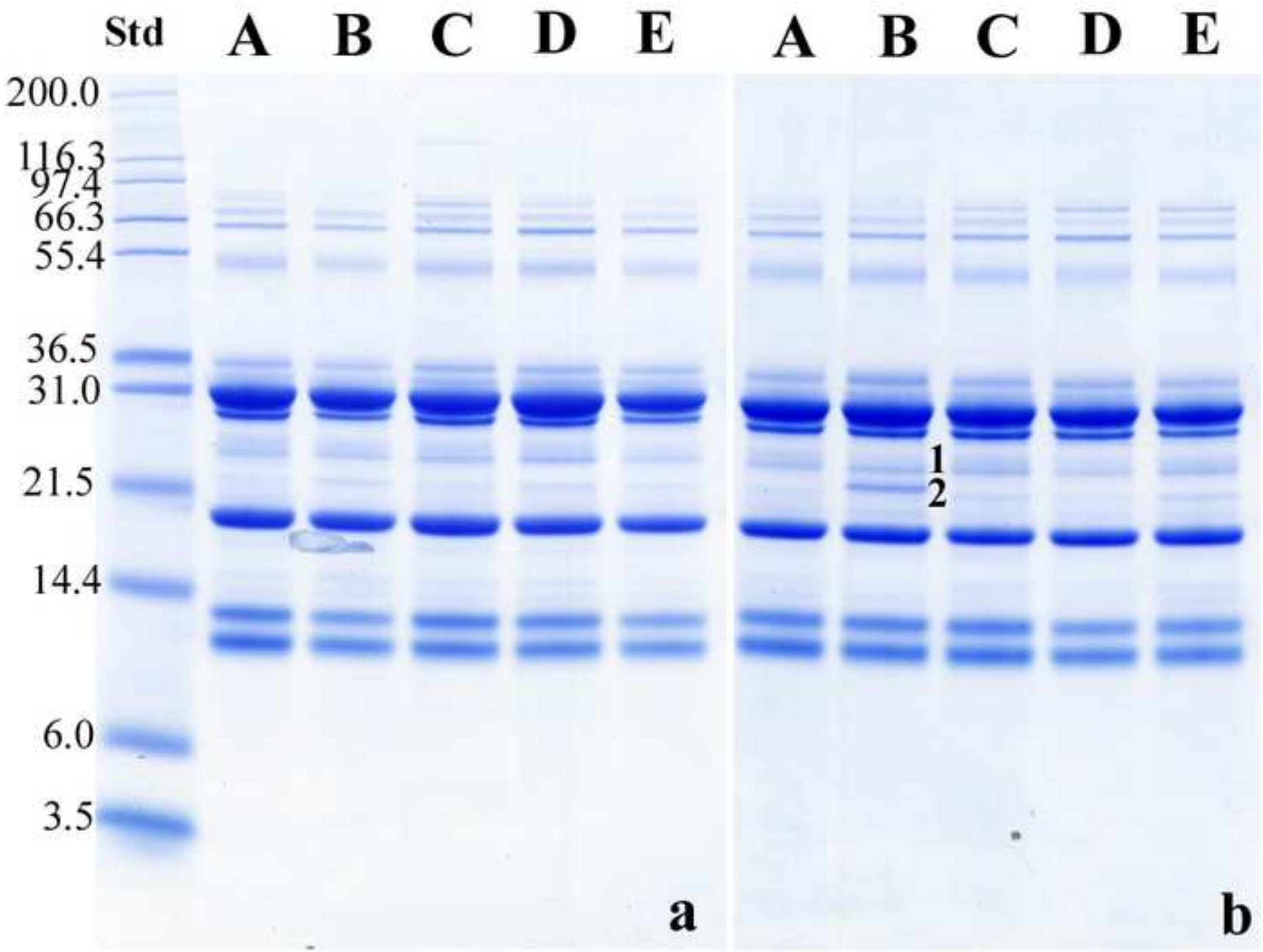
	N-term sequence	**Identified protein**	**Accession (NCBI)**	**Source organism**	**Theoretical mass (kDa)**
Band 1	T-N-I-P-Q-T-M-Q	Beta lactoglobulin **1**	P13613.1	*Equus asinus*	18.53
	K-H-E-I-K-X-V-S	Alpha s2 casein B precursor	CAX65660	*Equus asinus*	16.72
Band 2	T-N-I-P-Q-T-M-Q	Beta lactoglobulin **1**	P13613.1	*Equus asinus*	18.53

Table 4. DM microbiological analysis results. The numbers represent mean values and standard deviations (n=3) for the three samplings (log cfu/mL).

		Total viable count (TVC)		Lactic acid bacteria count (LAB)	
		Day 0	*Day 5*	*Day 0*	*Day 5*
Farm A	*Mean*	5.77 [a]	7.32 [a]	4.34 [a]	5.15 [a]
	SD	0.45	0.13	0.40	0.10
	Median	5.85	7.30	4.23	5.11
Farm B	*Mean*	4.51 [ab]	6.22 [a]	3.61 [a]	4.00 [ab]
	SD	0.66	0.52	0.10	0.31
	Median	4.18	6.40	3.57	4.00
Farm C	*Mean*	5.27 [a]	7.39 [a]	4.01 [a]	4.78 [ab]
	SD	0.42	0.30	0.89	1.22
	Median	5.11	7.46	3.65	4.20
Farm D	*Mean*	2.84 [b]	3.64 [b]	2.69 [a]	2.62 [ab]
	SD	0.96	1.52	1.18	1.15
	Median	2.72	4.04	1.95	1.95
Farm E	*Mean*	4.05 [ab]	6.76 [a]	2.34 [a]	2.31 [b]
	SD	1.12	1.26	0.66	0.62
	Median	4.00	7.41	1.95	1.95
Overall	*Mean*	5.38	7.21	4.20	5.19
	Median	4.86	7.00	3.57	4.00

[a-b] Different superscript letters in a column indicate significant differences according to Tukey's post-hoc test for TVC and to Dunnet-C test for LAB.

Figure 1


Std
A
B
C
D
E
A
B
C
D
E
200.0
116.3
97.4
66.3
55.4
36.5
31.0
21.5
14.4
6.0
3.5
1
2
a
b